

GENETIC CONTROL OF PLANT GROWTH AND DEVELOPMENT

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This invention relates to the genetic control of growth and/or development of plants and the cloning and expression of genes involved therein. More particularly, the invention relates to the cloning and expression of the Rht gene of *Triticum Aestivum*, and homologues from other species, and use of the genes in plants.

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- 10 An understanding of the genetic mechanisms which influence growth and development of plants, including flowering, provides a means for altering the characteristics of a target plant. Species for which manipulation of growth and/or development characteristics may be advantageous includes all
- 15 crops, with important examples being the cereals, rice and maize, probably the most agronomically important in warmer climatic zones, and wheat, barley, oats and rye in more temperate climates. Important crops for seed products are oil seed rape and canola, maize, sunflower, soyabean and
- 20 sorghum. Many crops which are harvested for their roots are, of course, grown annually from seed and the production of seed of any kind is very dependent upon the ability of the plant to flower, to be pollinated and to set seed. In horticulture, control of the timing of growth and
- 25 development, including flowering, is important.
- Horticultural plants whose flowering may be controlled include lettuce, endive and vegetable brassicas including cabbage, broccoli and cauliflower, and carnations and

geraniums. Dwarf plants on the one hand and over-size, taller plants on the other may be advantageous and/or desirable in various horticultural and agricultural contexts, further including trees, plantation crops and grasses.

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Recent decades have seen huge increases in wheat grain yields due to the incorporation of semi-dwarfing *Rht* homeoalleles into breeding programmes. These increases have enabled wheat productivity to keep pace with the demands of the rising world population. Previously, we described the cloning of the *Arabidopsis gai* alleles (International patent application PCT/GB97/00390 filed 12 February 1997 and published as WO97/29123 on 14 August 1998, John Innes Centre Innovations Limited, the full contents of which are incorporated herein by reference) which, like *Rht* mutant alleles in wheat (a monocot), confers a semi-dominant dwarf phenotype in *Arabidopsis* (a dicot) and a reduction in responsiveness to the plant growth hormone gibberellin (GA). *gai* encodes a mutant protein (*gai*) which lacks a 17 amino acid residue segment found near the N-terminus of the wild-type (GAI) protein. The sequence of this segment is highly conserved in a rice cDNA sequence (EST). Here we show that this cDNA maps to a short section of the overlapping cereal genome maps known to contain the *Rht* loci, and that we have used the cDNA to isolate the *Rht* genes of wheat. That genomes as widely diverged as those of *Arabidopsis* and *Triticum* should carry a conserved sequence which, when mutated, affects GA responsiveness, indicates a role for that sequence in GA

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signalling that is conserved throughout the plant kingdom. Furthermore, cloning of *Rht* permits its transfer to many different crop species, with the aim of yield enhancement as great as that obtained previously with wheat.

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The introduction of semi-dwarfing *Rht* homeoalleles (originally known as Norin 10 genes, derived from a Japanese variety, Norin 10) into elite bread-wheat breeding lines was one of the most significant contributors to the so-called "green revolution" (Gale et al, 1985. Dwarfing genes in wheat. In: Progress in Plant Breeding, G.E. Russell (ed) Butterworths, London pp 1-35). Wheat containing these homeoalleles consistently out-yield wheats lacking them, and now comprise around 80% of the world's wheat crop. The biological basis of this yield-enhancement appears to be two-fold. Firstly, the semi-dwarf phenotype conferred by the *Rht* alleles causes an increased resistance to lodging (flattening of plants by wind/rain with consequent loss of yield). Secondly, these alleles cause a reallocation of photoassimilate, with more being directed towards the grain, and less towards the stem (Gale et al, 1985). These properties have major effects on wheat yields, as demonstrated by the fact that UK wheat yields increased by over 20% during the years that *Rht*-containing lines were taken up by farmers.

The *rht* mutants are dwarfed because they contain a genetically dominant, mutant *rht* allele which compromises

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their responses to gibberellin (GA, an endogenous plant growth regulator) (Gale et al, 1976. Heredity 37; 283-289). Thus the coleoptiles of *rht* mutants, unlike those of wild-type wheat plants, do not respond to GA applications. In addition, *rht* mutants accumulate biologically active GAs to higher levels than found in wild-type controls (Lenton et al, 1987. Gibberellin insensitivity and depletion in wheat - consequences for development. In: Hormone action in Plant Development - a critical appraisal. GV Haod, JR Lenton, MB Jackson and RK Atkin (eds) Butterworths, London pp 145-160). These properties (genetic dominance, reduced GA-responses, and high endogenous GA levels) are common to the phenotypes conferred by mutations in other species (*D8/D9* in maize; *gai* in *Arabidopsis*), indicating that these mutant alleles define orthologous genes in these different species, supported further by the observation that *D8/D9* and *Rht* are syntenous loci in the genomes of maize and wheat.

According to a first aspect of the present invention there is provided a nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide with *Rht* function. The term "Rht function" indicates ability to influence the phenotype of a plant like the *Rht* gene of *Triticum*. "Rht function" may be observed phenotypically in a plant as inhibition, suppression, repression or reduction of plant growth which inhibition, suppression, repression or reduction is antagonised by GA. *Rht* expression tends to confer a dwarf phenotype on a plant which is antagonised by GA.

Overexpression in a plant from a nucleotide sequence encoding a polypeptide with *Rht* function may be used to confer a dwarf phenotype on a plant which is correctable by treatment with GA.

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Also according to an aspect of the present invention there is provided a nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide with ability to confer a *rht* mutant phenotype upon expression. *rht* mutant plants are
10 dwarfed compared with wild-type, the dwarfing being GA-insensitive.

Herein, "Rht" (capitalised) is used to refer to the wild-type function, while "rht" (uncapitalised) is used to refer to
15 mutant function.

Many plant growth and developmental processes are regulated by specific members of a family of tetracyclic diterpenoid growth factors known as gibberellins (GA) (Hooley, *Plant Mol.*
20 *Biol.* 26, 1529-1555 (1994)). By gibberellin or GA is meant a diterpenoid molecule with the basic carbon-ring structure shown in Figure 5 and possessing biological activity, i.e. we refer to biologically active gibberellins.

25 Biological activity may be defined by one or more of stimulation of cell elongation, leaf senescence or elicitation of the cereal aleurone α -amylase response. There are many standard assays available in the art, a positive

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result in any one or more of which signals a test gibberellin as biologically active (Hoad et al., *Phytochemistry* 20, 703-713 (1981); Serebryakov et al., *Phytochemistry* 23, 1847-1854 (1984); Smith et al., *Phytochemistry* 33, 17-20 (1993)).

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Assays available in the art include the lettuce hypocotyl assay, cucumber hypocotyl assay, and oat first leaf assay, all of which determine biological activity on the basis of ability of an applied gibberellin to cause elongation of the
10 respective tissue. Preferred assays are those in which the test composition is applied to a gibberellin-deficient plant. Such preferred assays include treatment of dwarf GA-deficient *Arabidopsis* to determine growth, the dwarf pea assay, in which internode elongation is determined, the Tan-ginbozu
15 dwarf rice assay, in which elongation of leaf sheath is determined, and the d5-maize assay, also in which elongation of leaf sheath is determined. The elongation bioassays measure the effects of general cell elongation in the respective organs and are not restricted to particular cell
20 types.

Further available assays include the dock (*Rumex*) leaf senescence assay and the cereal aleurone α -amylase assay. Aleurone cells which surround the endosperm in grain secrete
25 α -amylase on germination, which digests starch to produce sugars then used by the growing plant. The enzyme production is controlled by GA. Isolated aleurone cells given biologically active GA secrete α -amylase whose activity can

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then be assayed, for example by measurement of degradation of starch.

Structural features important for high biological activity (exhibited by GA₁, GA₃, GA₄ and GA₇) are a carboxyl group on C-6 of B-ring; C-19, C-10 lactone; and β -hydroxylation at C-3. β -hydroxylation at C-2 causes inactivity (exhibited by GA₈, GA₂₉, GA₃₄ and GA₅₁). *rht* mutants do not respond to GA treatment, e.g. treatment with GA₁, GA₃ or GA₄.

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Treatment with GA is preferably by spraying with aqueous solution, for example spraying with 10⁻⁴M GA₃ or GA₄ in aqueous solution, perhaps weekly or more frequently, and may be by placing droplets on plants rather than spraying. GA may be applied dissolved in an organic solvent such as ethanol or acetone, because it is more soluble in these than in water, but this is not preferred because these solvents have a tendency to damage plants. If an organic solvent is to be used, suitable formulations include 24 μ l of 0.6, 4.0 or 300mM GA₃ or GA₄ dissolved in 80% ethanol. Plants, e.g. *Arabidopsis*, may be grown on a medium containing GA, such as tissue culture medium (GM) solidified with agar and containing supplementary GA.

25 Nucleic acid according to the present invention may have the sequence of a wild-type *Rht* gene of *Triticum* or be a mutant, derivative, variant or allele of the sequence provided. Preferred mutants, derivatives, variants and alleles are

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those which encode a protein which retains a functional characteristic of the protein encoded by the wild-type gene, especially the ability for plant growth inhibition, which inhibition is antagonised by GA, or ability to confer on a plant one or more other characteristics responsive to GA treatment of the plant. Other preferred mutants, derivatives, variants and alleles encode a protein which confers a *rht* mutant phenotype, that is to say reduced plant growth which reduction is insensitive to GA, i.e. not overcome by GA treatment. Changes to a sequence, to produce a mutant, variant or derivative, may be by one or more of addition, insertion, deletion or substitution of one or more nucleotides in the nucleic acid, leading to the addition, insertion, deletion or substitution of one or more amino acids in the encoded polypeptide. Of course, changes to the nucleic acid which make no difference to the encoded amino acid sequence are included.

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A preferred nucleotide sequence for a *Rht* gene is one which encodes the RHT amino acid sequence shown in Figure 3b, especially a *Rht* coding sequence shown in Figure 3a. A preferred *rht* mutant lacks part or all of the 17 amino acid sequence underlined in Figure 3b, and/or part or the sequence DVAQKLEQLE, which immediately follows the 17 amino acid sequence underlined in Figure 3b.

Further preferred nucleotide sequences encode the amino acid sequence shown in any other figure herein, especially a

5 as shown in Figure 6a, 7a, 8a, 9a, 11a, 11c or 12a.

15 a plant cell, comprising nucleic acid according to the

A construct or vector comprising nucleic acid according to the present invention need not include a promoter or other regulatory sequence, particularly if the vector is to be used to introduce the nucleic acid into cells for recombination into the genome. However, in one aspect the present invention provides a nucleic acid construct comprising a *Rht*

or *rht* coding sequence (which includes homologues from other than *Triticum*) joined to a regulatory sequence for control of expression, the regulatory sequence being other than that naturally fused to the coding sequence and preferably of or
5 derived from another gene.

Nucleic acid molecules and vectors according to the present invention may be as an isolate, provided isolated from their natural environment, in substantially pure or homogeneous
10 form, or free or substantially free of nucleic acid or genes of the species of interest or origin other than the sequence encoding a polypeptide able to influence growth and/or development, which may include flowering, eg in *Triticum Aestivum* nucleic acid other than the *Rht* coding sequence.
15 The term "nucleic acid isolate" encompasses wholly or partially synthetic nucleic acid.

Nucleic acid may of course be double- or single-stranded, cDNA or genomic DNA, RNA, wholly or partially synthetic, as
20 appropriate. Of course, where nucleic acid according to the invention includes RNA, reference to the sequence shown should be construed as encompassing the RNA equivalent, with U substituted for T.

25 The present invention also encompasses the expression product of any of the nucleic acid sequences disclosed and methods of making the expression product by expression from encoding nucleic acid therefor under suitable conditions in suitable

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host cells. Those skilled in the art are well able to construct vectors and design protocols for expression and recovery of products of recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press. Transformation procedures depend on the host used, but are well known. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. Specific procedures and vectors previously used with wide success upon plants are described by Bevan, Nucl. Acids Res. (1984) 12, 8711-8721), and Guerineau and Mullineaux, (1993) Plant transformation and expression vectors. In: Plant Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148. The disclosures of Sambrook et al. and Ausubel et al. and all other documents mentioned herein are incorporated herein by reference.

Expression as a fusion with a polyhistidine tag allows purification of Rht or rht to be achieved using Ni-NTA resin

available from QIAGEN Inc. (USA) and DIAGEN GmbH (Germany).
See Janknecht et al., *Proc. Natl. Acad. Sci. USA* 88, 8972-
8976 (1991) and EP-A-0253303 and EP-A-0282042. Ni-NTA resin
has high affinity for proteins with consecutive histidines
5 close to the N- or C- terminus of the protein and so may be
used to purify histidine-tagged Rht or rht proteins from
plants, plant parts or extracts or from recombinant organisms
such as yeast or bacteria, e.g. *E. coli*, expressing the
protein.

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Purified Rht protein, e.g. produced recombinantly by
expression from encoding nucleic acid therefor, may be used
to raise antibodies employing techniques which are standard
in the art. Antibodies and polypeptides comprising antigen-
15 binding fragments of antibodies may be used in identifying
homologues from other species as discussed further below.

Methods of producing antibodies include immunising a mammal
(eg human, mouse, rat, rabbit, horse, goat, sheep or monkey)
20 with the protein or a fragment thereof. Antibodies may be
obtained from immunised animals using any of a variety of
techniques known in the art, and might be screened,
preferably using binding of antibody to antigen of interest.
For instance, Western blotting techniques or
25 immunoprecipitation may be used (Armitage et al, 1992, *Nature*
357: 80-82). Antibodies may be polyclonal or monoclonal.

As an alternative or supplement to immunising a mammal,

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antibodies with appropriate binding specificity may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, eg using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047.

Antibodies raised to a Rht, or rht, polypeptide can be used in the identification and/or isolation of homologous polypeptides, and then the encoding genes. Thus, the present invention provides a method of identifying or isolating a polypeptide with Rht function or ability to confer a rht mutant phenotype, comprising screening candidate polypeptides with a polypeptide comprising the antigen-binding domain of an antibody (for example whole antibody or a fragment thereof) which is able to bind an *Triticum Aestivum* Rht or rht polypeptide, or preferably has binding specificity for such a polypeptide, such as having the amino acid sequence shown in Figure 3b.

Candidate polypeptides for screening may for instance be the products of an expression library created using nucleic acid derived from a plant of interest, or may be the product of a purification process from a natural source.

A polypeptide found to bind the antibody may be isolated and then may be subject to amino acid sequencing. Any suitable technique may be used to sequence the polypeptide either

wholly or partially (for instance a fragment of the polypeptide may be sequenced). Amino acid sequence information may be used in obtaining nucleic acid encoding the polypeptide, for instance by designing one or more
5 oligonucleotides (e.g. a degenerate pool of oligonucleotides) for use as probes or primers in hybridisation to candidate nucleic acid, as discussed further below.

A further aspect of the present invention provides a method
10 of identifying and cloning *Rht* homologues from plant species other than *Triticum* which method employs a nucleotide sequence derived from any shown in Figure 2 or Figure 3a, or other figure herein. Sequences derived from these may themselves be used in identifying and in cloning other
15 sequences. The nucleotide sequence information provided herein, or any part thereof, may be used in a data-base search to find homologous sequences, expression products of which can be tested for *Rht* function. Alternatively, nucleic acid libraries may be screened using techniques well known to
20 those skilled in the art and homologous sequences thereby identified then tested.

For instance, the present invention also provides a method of identifying and/or isolating a *Rht* or *rht* homologue gene,
25 comprising probing candidate (or "target") nucleic acid with nucleic acid which encodes a polypeptide with *Rht* function or a fragment or mutant, derivative or allele thereof. The candidate nucleic acid (which may be, for instance, cDNA or

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genomic DNA) may be derived from any cell or organism which may contain or is suspected of containing nucleic acid encoding such a homologue.

5 In a preferred embodiment of this aspect of the present invention, the nucleic acid used for probing of candidate nucleic acid encodes an amino acid sequence shown in Figure 3b, a sequence complementary to a coding sequence, or a fragment of any of these, most preferably comprising a
10 nucleotide sequence shown in Figure 3a.

Alternatively, as discussed, a probe may be designed using amino acid sequence information obtained by sequencing a polypeptide identified as being able to be bound by an antigen-binding domain of an antibody which is able to bind a Rht or rht polypeptide such as one with the Rht amino acid sequence shown in Figure 3b.

Preferred conditions for probing are those which are
20 stringent enough for there to be a simple pattern with a
small number of hybridizations identified as positive which
can be investigated further. It is well known in the art to
increase stringency of hybridisation gradually until only a
few positive clones remain.

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As an alternative to probing, though still employing nucleic acid hybridisation, oligonucleotides designed to amplify DNA sequences from *Rht* genes may be used in PCR or other methods

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10 On the basis of amino acid sequence information,
oligonucleotide probes or primers may be designed, taking
into account the degeneracy of the genetic code, and, where
appropriate, codon usage of the organism from which the
candidate nucleic acid is derived. In particular, primers
15 and probes may be designed using information on conserved
sequences apparent from, for example, Figure 3 and/or Figure
4, also Figure 10.

Where a full-length encoding nucleic acid molecule has not
20 been obtained, a smaller molecule representing part of the
full molecule, may be used to obtain full-length clones.
Inserts may be prepared for example from partial cDNA clones
and used to screen cDNA libraries. The full-length clones
isolated may be subcloned into vectors such as expression
25 vectors or vectors suitable for transformation into plants.
Overlapping clones may be used to provide a full-length
sequence.

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Homology may be at the nucleotide sequence and/or amino acid sequence level. Preferably, the nucleic acid and/or amino acid sequence shares homology with the sequence encoded by the nucleotide sequence of Figure 3a, preferably at least about 50%, or 60%, or 70%, or 80% or 85% homology, most preferably at least 90%, 92%, 95% or 97% homology. Nucleic acid encoding such a polypeptide may preferably share with the *Triticum Rht* gene the ability to confer a particular phenotype on expression in a plant, preferably a phenotype which is GA responsive (i.e. there is a change in a characteristic of the plant on treatment with GA), such as the ability to inhibit plant growth where the inhibition is antagonised by GA. As noted, *Rht* expression in a plant may affect one or more other characteristics of the plant. A preferred characteristic that may be shared with the *Triticum Rht* gene is the ability to complement a *Rht* null mutant

phenotype in a plant such as *Triticum*, such phenotype being resistance to the dwarfing effect of paclobutrazol. The slender mutant of barley maps to a location in the barley genome equivalent to that of *Rht* in the wheat genome. Such 5 mutant plants are strongly paclobutrazol resistant. The present inventors believe that the slender barley mutant is a null mutant allele of the orthologous gene to wheat *Rht*, allowing for complementation of the barley mutant with the wheat gene. Ability to complement a slender mutant in barley 10 may be a characteristic of embodiments of the present invention.

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Some preferred embodiments of polypeptides according to the present invention (encoded by nucleic acid embodiments 15 according to the present invention) include the 17 amino acid sequence which is underlined in Figure 3b, or a contiguous sequence of amino acids residues with at least about 10 residues with similarity or identity with the respective corresponding residue (in terms of position) in 17 amino 20 acids which are underlined in Figure 3b, more preferably 11, 12, 13, 14, 15, 16 or 17 such residues, and/or the sequence DVAQKLEQLE, or a contiguous sequence of amino acids with at least about 5 residues with similarity or identity with the respective corresponding residue (in terms of position) 25 within DVAQKLEQLE, more preferably 6, 7, 8 or 9 such residues. Further embodiments include the 27 amino acid sequence DELLAAALGYKVRASDMADVAQKLEQLE, or a contiguous sequence of amino acids residues with at least about 15

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residues with similarity or identity with the respective corresponding residue (in terms of position) within this sequence, more preferably 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or 26 such residues.

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As is well-understood, homology at the amino acid level is generally in terms of amino acid similarity or identity. Similarity allows for "conservative variation", i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine. Similarity may be as defined and determined by the TBLASTN program, of Altschul et al. (1990) *J. Mol. Biol.* 215: 403-10, which is in standard use in the art, or more preferably GAP (Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, USA), which uses the algorithm of Needleman and Wunsch to align sequences. Suitable parameters for GAP include the default parameters, a gap creation penalty = 12 and gap extension penalty = 4, or gap creation penalty 3.00 and gap extension penalty 0.1. Homology may be over the full-length of the Rht sequence of Figure 3b, or may more preferably be over a contiguous sequence of 10 amino acids compared with DVAQKLEQLE, and/or a contiguous sequence of 17 amino acids, compared with the 17 amino acids underlined in Figure 3b, and/or a contiguous sequence of 27 amino acids compared with DELLAALGYKVRASDMADVAQKLEQLE, or a

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longer sequence, e.g. about 30, 40, 50 or more amino acids, compared with the amino acid sequence of Figure 3b and preferably including the underlined 17 amino acids and/or DVAQKLEQLE.

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At the nucleic acid level, homology may be over the full-length or more preferably by comparison with the 30 nucleotide coding sequence within the sequence of Figure 3a and encoding the sequence DVAQKLEQLE and/or the 51 nucleotide coding sequence within the sequence of Figure 3a and encoding the 17 amino acid sequence underlined in Figure 3b, or a longer sequence, e.g. about, 60, 70, 80, 90, 100, 120, 150 or more nucleotides and preferably including the 51 nucleotide of Figure 3 which encodes the underlined 17 amino acid sequence of Figure 3b.

As noted, similarity may be as defined and determined by the TBLASTN program, of Altschul et al. (1990) *J. Mol. Biol.* 215: 403-10, which is in standard use in the art, or the standard program BestFit, which is part of the Wisconsin Package, Version 8, September 1994, (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA, Wisconsin 53711). BestFit makes an optimal alignment of the best segment of similarity between two sequences. Optimal alignments are found by inserting gaps to maximize the number of matches using the local homology algorithm of Smith and Waterman (Adv. Appl. Math. (1981) 2: 482-489). Other algorithms include GAP, which uses the Needleman and Wunsch algorithm to

align two complete sequences that maximizes the number of matches and minimizes the number of gaps. As with any algorithm, generally the default parameters are used, which for GAP are a gap creation penalty = 12 and gap extension 5 penalty = 4. The algorithm FASTA (which uses the method of Pearson and Lipman (1988) *PNAS USA* 85: 2444-2448) is a further alternative.

Use of either of the terms "homology" and "homologous" herein 10 does not imply any necessary evolutionary relationship between compared sequences, in keeping for example with standard use of terms such as "homologous recombination" which merely requires that two nucleotide sequences are sufficiently similar to recombine under the appropriate 15 conditions. Further discussion of polypeptides according to the present invention, which may be encoded by nucleic acid according to the present invention, is found below.

The present invention extends to nucleic acid that hybridizes 20 with any one or more of the specific sequences disclosed herein under stringent conditions.

Hybridisation may be determined by probing with nucleic acid and identifying positive hybridisation under suitably 25 stringent conditions (in accordance with known techniques). For probing, preferred conditions are those which are stringent enough for there to be a simple pattern with a small number of hybridisations identified as positive which

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can be investigated further. It is well known in the art to increase stringency of hybridisation gradually until only a few positive clones remain.

- 5 Binding of a probe to target nucleic acid (e.g. DNA) may be measured using any of a variety of techniques at the disposal of those skilled in the art. For instance, probes may be radioactively, fluorescently or enzymatically labelled. Other methods not employing labelling of probe include
- 10 examination of restriction fragment length polymorphisms, amplification using PCR, RNAase cleavage and allele specific oligonucleotide probing.

- Probing may employ the standard Southern blotting technique.
- 15 For instance DNA may be extracted from cells and digested with different restriction enzymes. Restriction fragments may then be separated by electrophoresis on an agarose gel, before denaturation and transfer to a nitrocellulose filter. Labelled probe may be hybridised to the DNA fragments on the
- 20 filter and binding determined. DNA for probing may be prepared from RNA preparations from cells by techniques such as reverse-transcriptase- PRC.

- Preliminary experiments may be performed by hybridising under
- 25 low stringency conditions various probes to Southern blots of DNA digested with restriction enzymes. For probing, preferred conditions are those which are stringent enough for there to be a simple pattern with a small number of

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hybridisations identified as positive which can be investigated further. It is well known in the art to increase stringency of hybridisation gradually until only a few positive clones remain. Suitable conditions would be achieved when a large number of hybridising fragments were obtained while the background hybridisation was low. Using these conditions nucleic acid libraries, e.g. cDNA libraries representative of expressed sequences, may be searched. Those skilled in the art are well able to employ suitable conditions of the desired stringency for selective hybridisation, taking into account factors such as oligonucleotide length and base composition, temperature and so on.

For instance, screening may initially be carried out under conditions, which comprise a temperature of about 37°C or more, a formamide concentration of less than about 50%, and a moderate to low salt (e.g. Standard Saline Citrate ('SSC') = 0.15 M sodium chloride; 0.15 M sodium citrate; pH 7) concentration.

Alternatively, a temperature of about 50°C or more and a high salt (e.g. 'SSPE' = 0.180 mM sodium chloride; 9 mM disodium hydrogen phosphate; 9 mM sodium dihydrogen phosphate; 1 mM sodium EDTA; pH 7.4). Preferably the screening is carried out at about 37°C, a formamide concentration of about 20%, and a salt concentration of about 5 X SSC, or a temperature of about 50°C and a salt concentration of about 2 X SSPE. These

conditions will allow the identification of sequences which have a substantial degree of homology (similarity, identity) with the probe sequence, without requiring the perfect homology for the identification of a stable hybrid.

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Suitable conditions include, e.g. for detection of sequences that are about 80-90% identical, hybridization overnight at 42°C in 0.25M Na₂HPO₄, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 55°C in 0.1X SSC, 0.1% SDS. For

10 detection of sequences that are greater than about 90% identical, suitable conditions include hybridization overnight at 65°C in 0.25M Na₂HPO₄, pH 7.2, 6.5% SDS, 10% dextran sulphate and a final wash at 60°C in 0.1X SSC, 0.1% SDS.

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Conditions that may be used to differentiate Rht genes and homologues from others may include the following procedure:

First and second DNA molecules are run on an agarose gel, 20 blotted onto a membrane filter (Sambrook et al, 1989). The filters are incubated in prehybridization solution [6xSSC, 5x Denhart's solution, 20 mM Tris-HCl, 0.1% SDS, 2mM EDTA, 20 µg/ml Salmon sperm DNA] at 65°C for 5 hours, with constant shaking. Then, the solution is replaced with 30 ml of the 25 same, containing the radioactively-labelled second DNA (prepared according to standard techniques; see Sambrook et al, 1989), and incubated overnight at 65°C, with constant shaking. The following morning the filters are rinsed (one

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rinse with 3xSSC-0.1% SDS solution); and then washed: one wash at 65°C, for 25 minutes, with 3x SSC-0.1% SDS solution; and a second wash, at the same temperature and for the same time, with 0.1xSSC-0.1% SDS. Then the radioactive pattern on the filter is recorded using standard techniques (see Sambrook et al, 1989).

If need be, stringency can be increased by increasing the temperature of the washes, and/or reducing or even omitting altogether, the SSC in the wash solution.

(SSC is 150 mM NaCl, 15 mM sodium citrate. 50x Denhart's solution is 1% (w/v) ficoll, 1% polyvinylpyrrolidone, 1% (w/v) bovine serum albumin.)

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Homologues to *rht* mutants are also provided by the present invention. These may be mutants where the wild-type includes the 17 amino acids underlined in Figure 3b, or a contiguous sequence of 17 amino acids with at least about 10 (more preferably 11, 12, 13, 14, 15, 16 or 17) which have similarity or identity with the corresponding residue in the 17 amino acid sequence underlined in Figure 3, but the mutant does not. Similarly, such mutants may be where the wild-type includes DVAQKLEQLE or a contiguous sequence of 10 amino acids with at least about 5 (more preferably 6, 7, 8 or 9) which have similarity or identity with the corresponding residue in the sequence DVAQKLEQLE, but the mutant does not. Nucleic acid encoding such mutant polypeptides may on

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expression in a plant confer a phenotype which is insensitive or unresponsive to treatment of the plant with GA, that is a mutant phenotype which is not overcome or there is no reversion to wild-type phenotype on treatment of the plant with GA (though there may be some response in the plant on provision or depletion of GA).

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A further aspect of the present invention provides a nucleic acid isolate having a nucleotide sequence encoding a polypeptide which includes an amino acid sequence which is a mutant, allele, derivative or variant sequence of the *Rht* amino acid sequence of the species *Triticum Aestivum* shown in Figure 3b, or is a homologue of another species or a mutant, allele, derivative or variant thereof, wherein said mutant, allele, derivative, variant or homologue differs from the amino acid sequence shown in Figure 3b by way of insertion, deletion, addition and/or substitution of one or more amino acids, as obtainable by producing transgenic plants by transforming plants which have a *Rht* null mutant phenotype, which phenotype is resistance to the dwarfing effect of paclobutrazol, with test nucleic acid, causing or allowing expression from test nucleic acid within the transgenic plants, screening the transgenic plants for those exhibiting complementation of the *Rht* null mutant phenotype to identify test nucleic acid able to complement the *Rht* null mutant, deleting from nucleic acid so identified as being able to complement the *Rht* null mutant a nucleotide sequence encoding the 17 amino acid sequence underlined in Figure 3b or a

contiguous 17 amino acid sequence in which at least 10 residues have similarity or identity with the respective amino acid in the corresponding position in the 17 amino acid sequence underlined in Figure 3b, more preferably 11, 12, 13, 14, 15, 16 or 17, and/or a nucleotide sequence encoding DVAQKLEQLE or a contiguous sequence of 10 amino acids with at least about 5 (more preferably 6, 7, 8 or 9) which have similarity or identity with the corresponding residue in the sequence DVAQKLEQLE.

10

A cell containing nucleic acid of the present invention represents a further aspect of the invention, particularly a plant cell, or a bacterial cell.

15 The cell may comprise the nucleic acid encoding the protein by virtue of introduction into the cell or an ancestor thereof of the nucleic acid, e.g. by transformation using any suitable technique available to those skilled in the art.

20 Also according to the invention there is provided a plant cell having incorporated into its genome nucleic acid as disclosed.

Where a complete naturally occurring sequence is employed the plant cell may be of a plant other than the natural host of the sequence.

The present invention also provides a plant comprising such a

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plant cell.

Also according to the invention there is provided a plant cell having incorporated into its genome a sequence of 5 nucleotides as provided by the present invention, under operative control of a regulatory sequence for control of expression. A further aspect of the present invention provides a method of making such a plant cell involving introduction of a vector comprising the sequence of 10 nucleotides into a plant cell and causing or allowing recombination between the vector and the plant cell genome to introduce the sequence of nucleotides into the genome.

A plant according to the present invention may be one which 15 does not breed true in one or more properties. Plant varieties may be excluded, particularly registrable plant varieties according to Plant Breeders' Rights. It is noted that a plant need not be considered a "plant variety" simply because it contains stably within its genome a transgene, 20 introduced into a cell of the plant or an ancestor thereof.

In addition to a plant, the present invention provides any clone of such a plant, seed, selfed or hybrid progeny and descendants, and any part of any of these, such as cuttings, 25 seed. The invention provides any plant propagule, that is any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on. Also encompassed by the invention is a plant which is a sexually

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or asexually propagated off-spring, clone or descendant of such a plant, or any part or propagule of said plant, off-spring, clone or descendant.

5 The invention further provides a method of influencing the characteristics of a plant comprising expression of a heterologous *Rht* or *rht* gene sequence (or mutant, allele, derivative or homologue thereof, as discussed) within cells of the plant. The term "heterologous" indicates that the
10 gene/sequence of nucleotides in question have been introduced into said cells of the plant, or an ancestor thereof, using genetic engineering, that is to say by human intervention, which may comprise transformation. The gene may be on an extra-genomic vector or incorporated, preferably stably, into
15 the genome. The heterologous gene may replace an endogenous equivalent gene, ie one which normally performs the same or a similar function in control of growth and/or development, or the inserted sequence may be additional to an endogenous gene. An advantage of introduction of a heterologous gene is
20 the ability to place expression of the gene under the control of a promoter of choice, in order to be able to influence gene expression, and therefore growth and/or development of the plant according to preference. Furthermore, mutants and derivatives of the wild-type gene may be used in place of the
25 endogenous gene. The inserted gene may be foreign or exogenous to the host cell, e.g. of another plant species.

The principal characteristic which may be altered using the

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present invention is growth.

According to the model of the *Rht* gene as a growth repressor, under-expression of the gene may be used to promote growth, at least in plants which have only one endogenous gene conferring *Rht* function (not for example *Arabidopsis* which has endogenous homologues which would compensate). This may involve use of anti-sense or sense regulation. Taller plants may be made by knocking out *Rht* or the relevant homologous gene in the plant of interest. Plants may be made which are resistant to compounds which inhibit GA biosynthesis, such as paclobutrazol, for instance to allow use of a GA biosynthesis inhibitor to keep weeds dwarf but let crop plants grow tall.

Over-expression of a *Rht* gene may lead to a dwarf plant which is correctable by treatment with GA, as predicted by the *Rht* repression model.

Since *rht* mutant genes are dominant on phenotype, they may be used to make GA-insensitive dwarf plants. This may be applied for example to any transformable crop-plant, tree or fruit-tree species. It may provide higher yield/reduced lodging like *Rht* wheat. In rice this may provide GA-insensitive rice resistant to the Bakane disease, which is a problem in Japan and elsewhere. Dwarf ornamentals may be of value for the horticulture and cut-flower markets. Sequence manipulation may provide for varying degrees of severity of dwarfing, GA-insensitive phenotype, allowing tailoring of the

degree of severity to the needs of each crop-plant or the wishes of the manipulator. Over-expression of *rht*-mutant sequences is potentially the most useful.

- 5 A second characteristic that may be altered is plant development, for instance flowering. In some plants, and in certain environmental conditions, a GA signal is required for floral induction. For example, GA-deficient mutant *Arabidopsis* plants grown under short day conditions will not
- 10 flower unless treated with GA: these plants do flower normally when grown under long day conditions. *Arabidopsis gai* mutant plants show delayed flowering under short day conditions: severe mutants may not flower at all. Thus, for instance by *Rht* or *rht* gene expression or over-expression,
- 15 plants may be produced which remain vegetative until given GA treatment to induce flowering. This may be useful in horticultural contexts or for spinach, lettuce and other crops where suppression of bolting is desirable.
- 20 The nucleic acid according to the invention may be placed under the control of an externally inducible gene promoter to place the *Rht* or *rht* coding sequence under the control of the user.
- 25 The term "inducible" as applied to a promoter is well understood by those skilled in the art. In essence, expression under the control of an inducible promoter is "switched on" or increased in response to an applied

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stimulus. The nature of the stimulus varies between promoters. Some inducible promoters cause little or undetectable levels of expression (or no expression) in the absence of the appropriate stimulus. Other inducible promoters cause detectable constitutive expression in the absence of the stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible promoter is increased in the presence of the correct stimulus. The preferable situation is where the level of expression increases upon application of the relevant stimulus by an amount effective to alter a phenotypic characteristic. Thus an inducible (or "switchable") promoter may be used which causes a basic level of expression in the absence of the stimulus which level is too low to bring about a desired phenotype (and may in fact be zero). Upon application of the stimulus, expression is increased (or switched on) to a level which brings about the desired phenotype.

Suitable promoters include the Cauliflower Mosaic Virus 35S (CaMV 35S) gene promoter that is expressed at a high level in virtually all plant tissues (Benfey et al, 1990a and 1990b); the maize glutathione-S-transferase isoform II (GST-II-27) gene promoter which is activated in response to application of exogenous safener (WO93/01294, ICI Ltd); the cauliflower meri 5 promoter that is expressed in the vegetative apical meristem as well as several well localised positions in the plant body, eg inner phloem, flower primordia, branching

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points in root and shoot (Medford, 1992; Medford et al, 1991) and the *Arabidopsis thaliana* *LEAFY* promoter that is expressed very early in flower development (Weigel et al, 1992).

5 The GST-II-27 gene promoter has been shown to be induced by certain chemical compounds which can be applied to growing plants. The promoter is functional in both monocotyledons and dicotyledons. It can therefore be used to control gene expression in a variety of genetically modified plants,
10 including field crops such as canola, sunflower, tobacco, sugarbeet, cotton; cereals such as wheat, barley, rice, maize, sorghum; fruit such as tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, and melons; and vegetables such as carrot, lettuce, cabbage and onion. The
15 GST-II-27 promoter is also suitable for use in a variety of tissues, including roots, leaves, stems and reproductive tissues.

Accordingly, the present invention provides in a further
20 aspect a gene construct comprising an inducible promoter operatively linked to a nucleotide sequence provided by the present invention, such as the *Rht* gene of *Triticum* a homologue from another plant species or any mutant, derivative or allele thereof. This enables control of
25 expression of the gene. The invention also provides plants transformed with said gene construct and methods comprising introduction of such a construct into a plant cell and/or induction of expression of a construct within a plant cell,

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by application of a suitable stimulus, an effective exogenous inducer. The promoter may be the GST-II-27 gene promoter or any other inducible plant promoter.

- 5 When introducing a chosen gene construct into a cell, certain considerations must be taken into account, well known to those skilled in the art. The nucleic acid to be inserted should be assembled within a construct which contains effective regulatory elements which will drive transcription.
- 10 There must be available a method of transporting the construct into the cell. Once the construct is within the cell membrane, integration into the endogenous chromosomal material either will or will not occur. Finally, as far as plants are concerned the target cell type must be such that
- 15 cells can be regenerated into whole plants.

Selectable genetic markers may be used consisting of chimaeric genes that confer selectable phenotypes such as resistance to antibiotics such as kanamycin, hygromycin,

20 phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones and glyphosate.

An aspect of the present invention is the use of nucleic acid according to the invention in the production of a transgenic

25 plant.

A further aspect provides a method including introducing the nucleic acid into a plant cell and causing or allowing

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incorporation of the nucleic acid into the genome of the cell.

Any appropriate method of plant transformation may be used to generate plant cells comprising nucleic acid in accordance with the present invention. Following transformation, plants may be regenerated from transformed plant cells and tissue.

Successfully transformed cells and/or plants, i.e. with the construct incorporated into their genome, may be selected following introduction of the nucleic acid into plant cells, optionally followed by regeneration into a plant, e.g. using one or more marker genes such as antibiotic resistance (see above).

15

Plants transformed with the DNA segment containing the sequence may be produced by standard techniques which are already known for the genetic manipulation of plants. DNA can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by *Agrobacterium* exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966, Green et al. (1987) *Plant Tissue and Cell Culture*, Academic Press), electroporation (EP 290395, WO 8706614 Gelvin Debeyser - see attached) other forms of direct DNA uptake (DE 4005152, WO 9012096, US

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Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous species. Recently, there has been substantial progress towards the routine production of stable, fertile transgenic plants in almost all economically relevant monocot plants (Toriyama, et al. (1988) *Bio/Technology* 6, 1072-1074; Zhang, et al. (1988) *Plant Cell Rep.* 7, 379-384; Zhang, et al. (1988) *Theor Appl Genet* 76, 835-840; Shimamoto, et al. (1989) *Nature* 338, 274-276; Datta, et al. (1990) *Bio/Technology* 8, 736-740; Christou, et al. (1991) *Bio/Technology* 9, 957-962; Peng, et al. (1991) International Rice Research Institute, Manila, Philippines 563-574; Cao, et al. (1992) *Plant Cell Rep.* 11, 585-591; Li, et al. (1993) *Plant Cell Rep.* 12, 250-255; Rathore, et al. (1993) *Plant Molecular Biology* 21, 871-884; Fromm, et al. (1990) *Bio/Technology* 8, 833-839; Gordon-Kamm, et al. (1990) *Plant Cell* 2, 603-618; D'Halluin, et al. (1992) *Plant Cell* 4, 1495-1505; Walters, et al. (1992) *Plant Molecular Biology* 18, 189-200; Koziel, et al. (1993) *Biotechnology* 11, 194-200; Vasil, I. K. (1994) *Plant Molecular Biology* 25, 925-937; Weeks, et al. (1993) *Plant Physiology* 102, 1077-1084; Somers, et al. (1992) *Bio/Technology* 10, 1589-1594; WO92/14828). In particular, *Agrobacterium* mediated transformation is now

emerging also as an highly efficient transformation method in monocots (Hiei et al. (1994) *The Plant Journal* 6, 271-282).

The generation of fertile transgenic plants has been achieved in the cereals rice, maize, wheat, oat, and barley (reviewed in Shimamoto, K. (1994) *Current Opinion in Biotechnology* 5, 158-162.; Vasil, et al. (1992) *Bio/Technology* 10, 667-674; Vain et al., 1995, *Biotechnology Advances* 13 (4): 653-671; Vasil, 1996, *Nature Biotechnology* 14 page 702).

10

Microprojectile bombardment, electroporation and direct DNA uptake are preferred where *Agrobacterium* is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, eg bombardment with *Agrobacterium* coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with *Agrobacterium* (EP-A-486233).

20 *Brassica napus* transformation is described in Moloney et al. (1989) *Plant Cell Reports* 8: 238-242.

Following transformation, a plant may be regenerated, e.g. from single cells, callus tissue or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues and organs of the plant. Available techniques are reviewed in Vasil et al., *Cell Culture and Somatic Cell Genetics of Plants, Vol I, II and*

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III, *Laboratory Procedures and Their Applications*, Academic Press, 1984, and Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989.

- 5 The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention, nor is the choice of technique for plant regeneration.
- 10
- 15 In the present invention, over-expression may be achieved by introduction of the nucleotide sequence in a sense orientation. Thus, the present invention provides a method of influencing a characteristic of a plant, the method comprising causing or allowing expression of nucleic acid according to the invention from that nucleic acid within cells of the plant.
- 20

Under-expression of the gene product polypeptide may be achieved using anti-sense technology or "sense regulation".

- 25 The use of anti-sense genes or partial gene sequences to down-regulate gene expression is now well-established. DNA is placed under the control of a promoter such that transcription of the "anti-sense" strand of the DNA yields

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RNA which is complementary to normal mRNA transcribed from the "sense" strand of the target gene. For double-stranded DNA this is achieved by placing a coding sequence or a fragment thereof in a "reverse orientation" under the control of a promoter. The complementary anti-sense RNA sequence is thought then to bind with mRNA to form a duplex, inhibiting translation of the endogenous mRNA from the target gene into protein. Whether or not this is the actual mode of action is still uncertain. However, it is established fact that the technique works. See, for example, Rothstein et al, 1987; Smith et al, (1988) *Nature* 334, 724-726; Zhang et al, (1992) *The Plant Cell* 4, 1575-1588, English et al., (1996) *The Plant Cell* 8, 179-188. Antisense technology is also reviewed in reviewed in Bourque, (1995), *Plant Science* 105, 125-149, and Flavell, (1994) *PNAS USA* 91, 3490-3496.

The complete sequence corresponding to the coding sequence in reverse orientation need not be used. For example fragments of sufficient length may be used. It is a routine matter for the person skilled in the art to screen fragments of various sizes and from various parts of the coding sequence to optimise the level of anti-sense inhibition. It may be advantageous to include the initiating methionine ATG codon, and perhaps one or more nucleotides upstream of the initiating codon. A further possibility is to target a regulatory sequence of a gene, e.g. a sequence that is characteristic of one or more genes in one or more pathogens against which resistance is desired. A suitable fragment

may have at least about 14-23 nucleotides, e.g. about 15, 16 or 17, or more, at least about 25, at least about 30, at least about 40, at least about 50, or more. Other fragments may be at least about 300 nucleotides, at least about 400
5 nucleotides, at least about 500 nucleotides, at least about 600 nucleotides, at least about 700 nucleotides or more. Such fragments in the sense orientation may be used in co-suppression (see below).

10 Total complementarity of sequence is not essential, though may be preferred. One or more nucleotides may differ in the anti-sense construct from the target gene. It may be preferred for there to be sufficient homology for the respective anti-sense and sense RNA molecules to hybridise,
15 particularly under the conditions existing in a plant cell.

Thus, the present invention also provides a method of influencing a characteristic of a plant, the method comprising causing or allowing anti-sense transcription from
20 nucleic acid according to the invention within cells of the plant.

When additional copies of the target gene are inserted in sense, that is the same, orientation as the target gene, a
25 range of phenotypes is produced which includes individuals where over-expression occurs and some where under-expression of protein from the target gene occurs. When the inserted gene is only part of the endogenous gene the number of

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under-expressing individuals in the transgenic population increases. The mechanism by which sense regulation occurs, particularly down-regulation, is not well-understood.

However, this technique is also well-reported in scientific and patent literature and is used routinely for gene control. See, for example, van der Krol et al., (1990) *The Plant Cell* 2, 291-299; Napoli et al., (1990) *The Plant Cell* 2, 279-289; Zhang et al., (1992) *The Plant Cell* 4, 1575-1588, and US-A-5,231,020.

10

Thus, the present invention also provides a method of influencing a characteristic of a plant, the method comprising causing or allowing expression from nucleic acid according to the invention within cells of the plant. This may be used to influence growth.

Aspects and embodiments of the present invention will now be illustrated, by way of example, with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

BRIEF DESCRIPTION OF THE DRAWING

~~The following figures are included herein:~~

25 ~~Figure 1: Alignment of N-terminus predicted GAI amino acid sequence (Gai) with rice EST D39460 (0830), with a region of homology outlined in black.~~

See
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Sub 85 > Figure 2: DNA sequences from C15-1, 14a1 and 5a1.

Sub 05 > Figure 2a shows a consensus DNA sequence cDNA C15-1 (obtained via single-pass sequencing).

5

Sub C6 > Figure 2b shows data from original DNA sequencing runs from 14a1 (single-pass).

Sub C7 > Figure 2c shows data from original DNA sequencing runs from 10 5a1 (single-pass).

Sub C6 > Figure 3: Rht sequences.

Sub C8 > Figure 3a shows a composite DNA sequence of wheat Rht gene 15 derived from data in Figure 2, including coding sequence.

Sub C9 > Figure 3b shows an alignment of the entire predicted Rht protein sequence encoded by the coding sequence of Figure 2 (rht) with the entire predicted GAI protein sequence of 20 *Arabidopsis* (Gai). Regions of sequence identity are highlighted in black.

Sub E7 > Figure 4: D39460 sequence.

Sub C10 > 25 Figure 4a shows DNA sequence (single-pass) of rice cDNA D39460. This cDNA is an incomplete, partial clone, missing the 3' end of the mRNA from which it is derived.

Sub C11 > Figure 4b shows alignment of the entire predicted Rht protein sequence (wheat - encoded by the coding sequence of Figure 2) with that of GAI (Gai) and rice protein sequence predicted from DNA sequence in Figure 4a (Rice). Regions of amino acid identity are highlighted in black; some conservative substitutions are shaded.

Figure 5: The basic carbon-ring structure of gibberellins.

Sub E8 > 10 Figure 6: Rice EST sequence

Sub C12 > Figure 6a shows the nucleotide sequence of rice EST D39460, as determined by the present inventors.

Sub C13 > 15 Figure 6b shows the predicted amino acid sequence encoded by the rice EST sequence of Figure 6a.

Sub E9 > Figure 7: Wheat C15-1 cDNA

Sub C14 > 20 Figure 7a shows the nucleotide sequence of the wheat C15-1 cDNA.

Sub C15 > Figure 7b shows the predicted amino acid sequence of the wheat C15-1 cDNA of Figure 7a.

25

Sub E10 > Figure 8: Wheat 5a1 genomic clone

Sub C16 > Figure 8a shows the nucleotide sequence of the 5a1 wheat

genomic clone.

Figure 8b shows the predicted amino acid sequence of the 5a1 wheat genomic clone of Figure 8a.

5

Figure 9: \ Maize 1a1 genomic clone

Figure 9a shows the nucleotide sequence of the 1a1 maize genomic clone, i.e. *D8*.

10

Figure 9b shows the amino acid sequence of the maize 1a1 genomic clone of Figure 9a.

Figure 10 shows a PRETTYBOX alignment of amino acid sequences of the maize D8 polypeptide with, the wheat Rht polypeptide the rice EST sequence determined by the present inventors and the *Arabidopsis thaliana* Gai polypeptide.

Figure 11: Sequences of maize *D8* alleles

20

Figure 11a shows a partial nucleotide sequence of the maize D8-1 allele.

Figure 11b shows a partial amino acid sequence of the maize D8-1 allele.

Figure 11c shows a partial nucleotide sequence of the maize D8-2023 allele.

Sub C23
Figure 11d shows a partial amino-acid sequence of the maize D8-2023 allele.

Sub E13
Figure 12: Wheat *rht-10* allele

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Sub C24
Figure 12a shows a partial nucleotide sequence of the wheat *rht-10* allele.

Sub C25
Figure 12b shows a partial amino acid sequence of the wheat *rht-10* allele.

Sub C26
Previously, we cloned the *GAI* gene of *Arabidopsis* (PCT/GB97/00390 - WO97/29123 published 14 August 1997).

15 Comparison of the DNA sequences of the wild-type (*GAI*) and mutant (*gai*) alleles showed that *gai* encodes a mutant predicted protein product (*gai*) which lacks a segment of 17 amino acids from close to the N-terminus of the protein. Screening of the DNA sequence databases with the *GAI* sequence
20 revealed the existence of a rice EST (D39460) which contains a region of sequence very closely related to that of the segment that is deleted from *GAI* in the *gai* protein. A comparison of the predicted amino acid sequences from the region DELLA to EQLE are identical in both sequences. The
25 two differences (V/A; E/D) are conservative substitutions, in which one amino acid residue is replaced by another having very similar chemical properties. In addition, the region of identity extends beyond the boundary of the deletion region

in the gai protein. The sequence DVAQKLEQLE is not affected by the deletion in gai, and yet is perfectly conserved between the GAI and D39460 sequences (Figure 1).

Sub
C2
C15-1

5 An approximately 700 bp *SalI*-*NotI* subfragment of D39460 was used in low-stringency hybridization experiments to isolate hybridizing clones from wheat cDNA and genomic libraries (made from DNA from the variety Chinese Spring) and from a maize genomic library (made from line B73N). Several wheat
10 clones were isolated, including C15-1 and C15-10 (cDNAs), and 5a1 and 14a1 (genomic clones). Clone C15-1 has been used in gene mapping experiments. Nullisomic-tetrasomic analysis showed that clone C15-1 hybridizes to genomic DNA fragments derived from wheat chromosomes 4A, 4B and 4D. This is
15 consistent with clone C15-1 containing *Rht* sequence, since the *Rht* loci map to the group 4 chromosomes. Furthermore, recombinant analysis using a population segregating for the *Rht-D1b* (formerly *Rht2*) allele identified a hybridizing fragment that displayed perfect co-segregation with the
20 mutant allele. This placed the genomic location of the gene encoding the mRNA sequence in cDNA C15-1 within a 2 cM segment (that was already known to contain *Rht*) of the group 4 chromosomes, and provides strong evidence that the cDNA and genomic clones do indeed contain the *Rht* gene. The maize D8
25 DNA sequence disclosed herein is from subcloned contiguous 1.8 kb and 3.0 kb *SalI* fragments (cloned into Bluescript™ SK+) from 1a1. The wheat *Rht* sequence disclosed herein is from a 5.7 kb *DraI* subfragment cloned into Bluescript™ SK+)

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from clone 5a1.

Figure 2a gives the complete (single-pass) DNA sequence of cDNA C15-1. We have also obtained DNA sequence for C15-10; it is identical with that of C15-1, and is therefore not shown. Figures 2b and 2c show original data from individual sequencing runs from clones 14a1 and 5a1. The sequences shown in Figure 2 can be overlapped to make a composite DNA sequence, shown in Figure 3a. This sequence displays strong homology with that of *Arabidopsis GAI*, as revealed by a comparison of the amino acid sequence of a predicted translational product of the wheat sequence (*Rht*) with that of *GAI* (*GAI*), shown in Figure 3b. In particular, the predicted amino acid sequence of the presumptive *Rht* reveals a region of near-identity with *GAI* over the region that is missing in *gai* (Figure 4). Figure 4 reveals that the homology that extends beyond the *gai* deletion region in the rice EST is also conserved in *Rht* (DVAQKLEQLE), thus indicating that this region, in addition to that found in the *gai* deletion, is involved in GA signal-transduction. This region is not found in *SCR*, another protein that is related in sequence to *GAI* but which is not involved in GA signalling. The primers used in the above sequencing experiments are shown in Table 1.

25

Further confirmation that these sequences are indeed the wheat *Rht* and maize *D8* loci has been obtained by analysis of gene sequences from various mutant alleles, as follows.

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The present inventors obtained and sequenced the clone identified on the database as the rice EST D39460, and the nucleotide and predicted amino acid sequences resulting from that work are shown in Figure 6a and Figure 6b respectively.

5

Previous work on the *GAI* gene of *Arabidopsis* showed that the *GAI* protein consists of two sections, an N-terminal half displaying no homology with any protein of known function, and a C-terminal half displaying extensive homology with the *Arabidopsis* SCR candidate transcription factor (Peng et al. (1997) *Genes and Development* 11: 3194-3205; PCT/GB97/00390). As described above, deletion of a portion of the N-terminal half of the protein causes the reduced GA-responses characteristic of the *gai* mutant allele (Peng et al., 1997; PCT/GB97/00390). The inventors therefore predicted that if *D8* and *Rht* are respectively maize and wheat functional homologues (orthologues) of *Arabidopsis* *GAI*, then dominant mutant alleles of *D8* and *Rht* should also contain mutations affecting the N-terminal sections of the proteins that they encode.

Previous reports describe a number of dominant mutant alleles at *D8* and at *Rht*, in particular *D8-1*, *D8-2023* and *Rht-D1c* (formerly *Rht10*) (Börner et al. (1996) *Euphytica* 89: 69-75; Harberd and Freeling (1989) *Genetics* 121: 827-838; Winkler and Freeling (1994) *Planta* 193: 341-348). The present inventors therefore cloned the candidate *D8/Rht* genes from these mutants, and examined by DNA sequencing the portion of

the gene that encodes the N-terminal half of the protein.

A fragment of the candidate *D8* or *Rht* genes that encodes a portion of the N-terminal half of the *D8/Rht* protein was amplified via PCR from genomic DNA of plants containing *D8-1*, *D8-2023* and *Rht-D1c*, using the following primers for amplification: for *D8-1*, primers ZM-15 and ZM-24; for *D8-2023*, primers ZM-9 and ZM-11; for *Rht-D1c*, nested PCR was performed using *Rht-15* and *Rht-26* followed by *Rht-16* and *Rha-*

2. PCR reactions were performed using a Perkin Elmer geneAmp XL PCR kit, using the following conditions: reactions were incubated at 94°C for 1 min, then subjected to 13 cycles of 94°C, 15 sec - x°C for 15 sec - 69°C 5 min (where x is reduced by 1°C per cycle starting at 64°C and finishing at 52°C), then 25 cycles of 94°C, 15 sec - 53°C, 15 sec - 65°C, 5 min, then 10 min at 70°C. These fragments were then cloned into the pGEM^R-T Easy vector (Promega, see Technical Manual), and their DNA sequences were determined.

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Mutations were found in the candidate *D8* and *Rht* genes in each of the above mutants. The *D8-1* mutation is an in-frame deletion which removes amino acids VAQK (55-59) and adds a G (see sequence in Figure 11a and Figure 11b). This deletion overlaps with the conserved DVAQKLEQLE homology block described above. *D8-2023* is another in-frame deletion mutation that removes amino acids LATDTVHYNPSD (87-98) from the N-terminus of the *D8* protein (see Figure 11c and Figure 11d). This deletion does not overlap with the deletion in

gai or D8-1, but covers another region that is highly conserved between GAI, D8 and Rht (see Figure 10). Finally, Rht-D1c contains another small in-frame deletion that removes amino acids LNAPPPPLPPAPQ (109-121) in the N-terminal region 5 of the mutant Rht protein that it encodes (see Figure 12a and Figure 12b) (LN-P is conserved between GAI, D8 and Rht, see Figure 10).

Thus all of the above described mutant alleles are dominant, 10 and confer dwarfism associated with reduced GA-response. All three of these alleles contain deletion mutations which remove a portion of the N-terminal half of the protein that they encode. These observations demonstrate that the D8 and Rht genes of maize and wheat have been cloned.

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TABLE 1 - Primers used in the sequencing of Rht

<u>Name</u>		<u>Sequence</u>	
		<u>Sense</u>	
5	15-L	TTTGCGCCAATTATTGGCCAGAGATAGATAGAGAG	Forward
	16-L	GTGGCGGCATGGGTTCGTCCGAGGACAAGATGATG	Forward
	23-L	CATGGAGGCGGTGGAGAACTGGGAACGAAGAAGGG	Reverse
	26-L	CCCGGCCAGGCGCCATGCCGAGGTGGCAATCAGGG	Reverse
10	3-L	GGTATCTGCTTCACCAGCGCCTCCGCGGCGGAGAG	Reverse
	9-L	ATCGGCCGCGAGCGCGTAGATGCTGCTGGAGGAGTC	Reverse
	RHA-1	CTGGTGAAGCAGATACCCTTGC	Forward
	RHA-2	CTGGTTGGCGGTGAAGTGCG	Reverse
	RHA-3	GCAAGGGTATCTGCTTCACCAGC	Reverse
15	RHA-5	CGCACTTCACCGCCAACCAG	Forward
	RHA-6	TTGTGATTTGCCCTCCTGTTTCC	Forward
	RHA-7	CCGTGCGCCCCCGTGCGGCCCCAG	Forward
	RHA-8	AGGCTGCCTGACGCTGGGGTTGC	Forward
	RHT-9	GATCGGCCGCGAGCGCGTAGATGC	Reverse
20	RHT-10	GATCCCGCACGGAGTCGGCGGACAG	Reverse
	RHT-12	TCCGACAGCATGCTCTCGACCCAAG	Reverse
	RHT-13	TTCCGTCCGTCTGGCGTGAAGAGG	Forward
	RHT-14	AAATCCCGAACC CGCCCCCAGAAC	Forward
	RHT-15	GCGCCAATTATTGGCCAGAGATAG	Forward
25	RHT-16	GGCATGGGTTCGTCCGAGGACAAG	Forward
	RHT-18	TTGTCCTCGGACGAACCCATGCCG	Reverse
	RHT-19	GATCCAAATCCCGAACC CGCCC	Forward
	RHT-20	GTAGATGCTGCTGGAGGAGTCG	Reverse
	RHT-21	GTCGTCCATCCACCTCTTCACG	Reverse
30	RHT-22	GCCAGAGATAGATAGAGAGGCG	Forward
	RHT-23	TAGGGCTTAGGAGTTTTACGGG	Reverse
	RHT-24	CGGAGTCGGCGGACAGGTCGGC	Reverse
	RHT-25	CGGAGAGGTTCTCCTGCTGCACGGC	Reverse
	RHT-26	TGTGCAACCCCGAGCGTCAGGCAG	Reverse
35	RHT-27	GCGGCCTCGTCGCCGCCACGCTC	Forward
	RHT-28	TGGCGGCGACGAGGCCGCGGTAC	Reverse
	RHT-29	AAGAATAAGGAAGAGATGGAGATGGTTG	Reverse
	RHT-30	TCTGCAACGTGGTGGCCTGCGAG	Forward
	RHT-31	CCCCTCGCAGGCCACACGTTGC	Reverse
40	RHT-32	TTGGGTCGAGAGCATGCTGTCCGAG	Forward

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Sub 28 > TABLE 2 - Primers used in the sequence of D-8 clones

	<u>Name</u>	<u>Sequence</u>	<u>Sense</u>
5	ZM-8	GGCGATGACACGGATGACG	Forward
	ZM-9	CTTGCGCATGGCACC GCCCTGCGACGAAG	Reverse
	ZM-10	CCAGCTAATAATGGCTTGCGCGCCTCG	Reverse
	ZM-11	TATCCCAGAACCGAAACCGAG	Forward
	ZM-12	CGGCGTCTTGGTACTCGCGCTTCATG	Reverse
10	ZM-13	TGGGCTCCCGCGCCGAGTCCGTGGAC	Reverse
	ZM-14	CTCCAAGCCTCTTGCGCTGACCGAGATCGAG	Forward
	ZM-15	TCCACAGGCTCACCAGTCACCAACATCAATC	Forward
	ZM-16	ACGGTACTGGAAGTCCACGCGGATGGTGTG	Reverse
	ZM-17	CGCACACCATCCGCGTGGACTTCCAGTAC	Forward
15	ZM-18	CTCGGCCGGCAGATCTGCAACGTGGTG	Forward
	ZM-19	TTGTGACGGTGGACGATGTGGACGCGAGCCTTG	Reverse
	ZM-20	GGACGCTGCGACAAACCGTCCATCGATCCAAC	Forward
	ZM-21	TCCGAAATCATGAAGCGCGAGTACCAAGAC	Forward
	ZM-22	TCGGGTACAAGGTGCGTTCGTCGGATATG	Forward
20	ZM-23	ATGAAGCGCGAGTACCAAGAC	Forward
	ZM-24	GTGTGCCTTGATGCGGTCCAGAAG	Reverse
	ZM-25	AACCACCCCTCCCTGATCACGGAG	Reverse
	ZM-27	CACTAGGAGCTCCGTGGTCTGAAGCTG	Forward
	ZM-28	GCTGCGCAAGAAGCCGGTGCAGCTC	Reverse
25	ZM-29	AGTACACTTCCGACATGACTTG	Reverse

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